N-Acetylcysteine and acute retinal laser lesion in the Colubrid snake eye.

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Abstract

This study examined the role of oxidative stress and the effect of a single dose treatment with N-Acetylcysteine (NAC) on the temporal development of acute laser-induced retinal injury. We used the snake eye/Scanning Laser Ophthalmoscope (SLO) model, an *in vivo,* non-invasive ocular imaging technique, which has the ability to image cellular retinal detail and allows for studying morphological changes of retinal injury over time. For this study 12 corn-snakes (*Elaphe g. guttata*) received 5 laser exposures per eye, followed by either a single dose of the antioxidant NAC (150mg/kg, IP in sterile saline) or placebo. Laser exposures were made with a Nd: VO_4 DPSS, 532nm laser, coaxially aligned to the SLO. Shuttered pulses were 20msec x 50 mW; 1mJ each. Retinal images were taken using a Rodenstock cSLO and were digitally recorded at 1, 6, 24-hrs, and at 3-wks post-exposure. Lesions were assessed by two raters blind to the conditions of the study yielding measures of damaged area and counts of missing or damaged photoreceptors. Treated eyes showed a significant beneficial effect overall, and these results suggest that oxidative stress plays a role in laser-induced retinal injury. The use of NAC or a similar antioxidant shows promise as a therapeutic tool.

Introduction

A role for oxidative stress (OS) in acute laser induced retinal injury has been long hypothesized.[1,2] In thermal laser retinal injury some of the cells affected are destroyed immediately. Many others, on the periphery of the lesion, are damaged and die later, in the subsequent hours or days. [3] The cells killed immediately are unlikely to so be saved by any possible treatment, but at least some of the cells that succumb to the delayed effects of the injury may be savable. Whether the mechanism of the delayed cell death is apoptotic or necrotic, and whether the OS is from thermal effects or an immuno-inflammatory response, antioxidant therapy might alter their fate. Using an antioxidant to treat laser retinal injury was the strategy behind this pilot study.

The confocal Scanning Laser Ophthalmoscope (cSLO) was used to evaluate retinal laser injury in the otherwise intact eye of a common species of non-venomous North American Colubrid snake, the Corn snake and its close relative, the Great Plains rat snake. The snake eye/Scanning Laser Ophthalmoscope (SLO) model, a non-invasive ocular imaging technique, has the ability to image cellular changes in the *in vivo* retina (Figure 1).[4,5] In particular the photoreceptor matrix is visible in detail and the fates of individual photoreceptors can be tracked across time. This unique ocular imaging capability gives the preparation potential for use as a longitudinal model in the study of laser retinal injury.

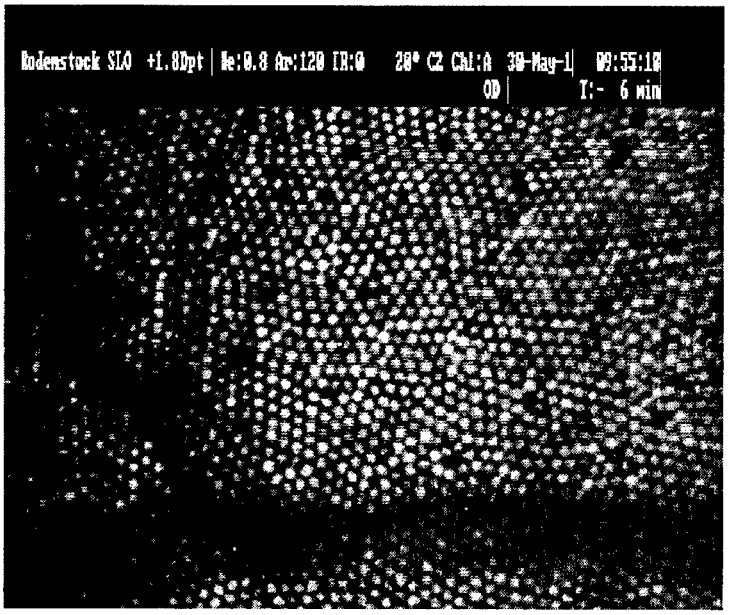


Figure 1
The photoreceptor matrix in the intact *in vivo* corn snake eye, imaged through the natural pupil, with a Scanning Laser Ophthalmoscope. This animal has an all cone retina, its cell spacing ~ 12 microns, similar in size to cones in human macula. Images of this detail are impossible in most animal eyes without sacrifice and histological preparation. In this model, the only prep is anesthesia and placement in front of the SLO. At different confocal planes, similarly detailed views of the retinal micro-circulation and nerve fiber are evident.

Laser Interaction with Tissue and Cells XV, edited by
Steven L. Jacques, William P. Roach, Proc. of SPIE Vol. 5319
(SPIE, Bellingham, WA, 2004) · 1605-7422/04/$15 · doi: 10.1117/12.530362

The animals we have chosen for this model are North American rat snakes of the genus *Elaphe,* particularly the corn-snake (*E. g. guttata*), and the closely related Great Plains rat snake (*E. g. emoryi*).[6] These species were chosen for their excellent ocular imaging potential, their gentle nature, ease of husbandry, general availability, and their common use in scientific studies of all kinds. In the process of developing this model for the study of laser retinal injury we are acquiring comparative knowledge about the snake visual system (Figure 2). [7,8]

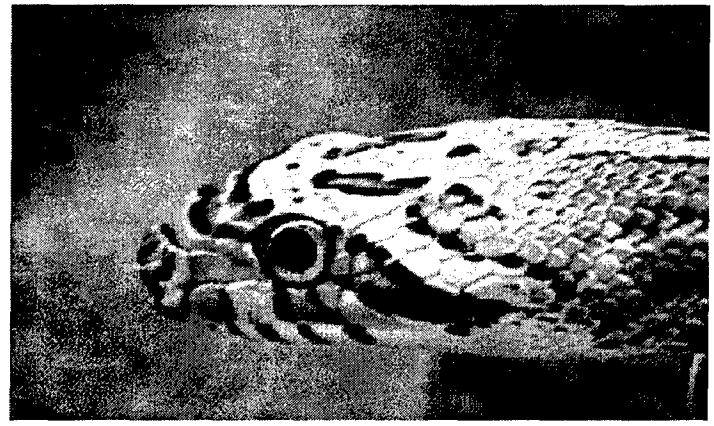


Figure 2 (above)
Great Plains rat snake: (*Elaphe g. emoryi*). Non-venomous and inoffensive, well suited for laboratory use.

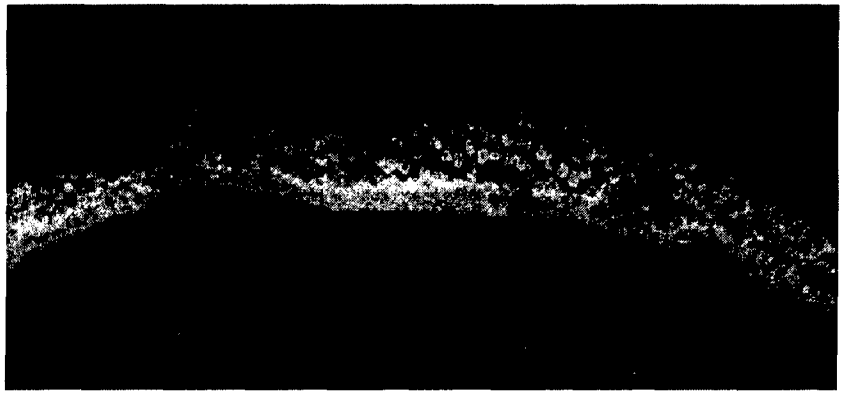


Figure 3
Corn snake retina. A histological preparation stained with fluorescent antibody specific for cones, upper layer.

The eye of the rat snake has an all cone retina. These cones are similar in size to cones in the human macula at approximately 8-10 micrometers in diameter (Figure 3). This differs from other common small-eyed animal models such as the rat and the mouse, as these have rod-dominated retinae. The ability to image the photoreceptors through the natural pupil in the snake eye was noticed long ago. [9,10] The SLO however, with its small beam size at the corneal plane and monochromatic (laser) illumination sources, allows detailed retinal imagery with relative ease.

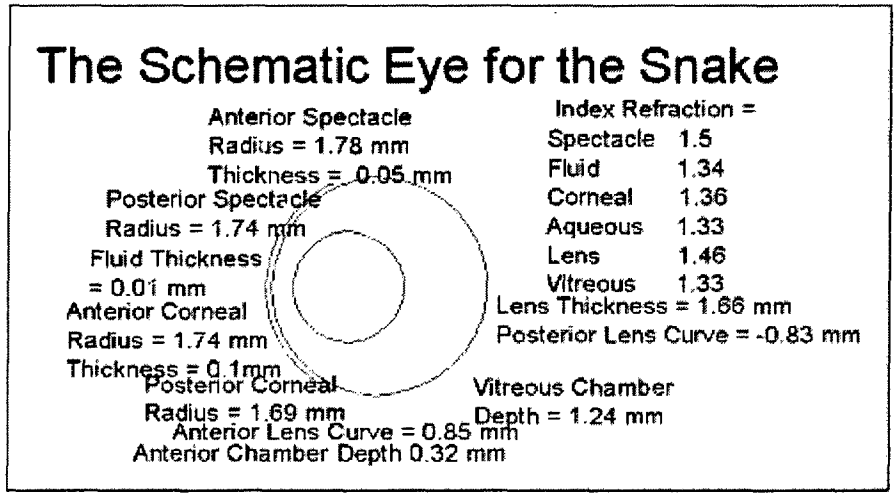


Figure 4
Colubrid snake eye: Dimensions and components. Note the presence of the spectacle; a clear, hard, dry optical first surface. Measurements listed here are from the eye of the Checkered garter snake, slightly smaller but qualitatively very similar to the rat snake eye.

The small size of the Colubrid snake eye, and its concomitant high optical power, along with its relatively large pupil, give it a high numerical aperture. Another feature, unique to small-eyed animal models, is the immovable eyelid, a modified scale called the spectacle. This serves as a hard, dry, optical first surface, contributing to lasting retinal image quality during anesthetized eye exams. Finally the small size of the snake eye produces a differential retinal area: visual angle ratio that magnifies retinal images relative to larger eyes. This suite of physical and optical properties allows detailed retinal imagery (Figure 4). [11]

The role of oxidative stress (OS) in acute laser induced retinal injury (and many other retinal disease/injuries) is not fully understood. In thermal laser retinal injury many of the cells affected are destroyed immediately (Figure 5A). Observing the temporal development of laser lesions in the snake eye, we noticed a group of photoreceptors on the boundary of a thermal lesion which show pathological changes shortly after the exposure, but which are not immediately destroyed. If followed for several weeks most of these cells appear to die (Figure 5B).

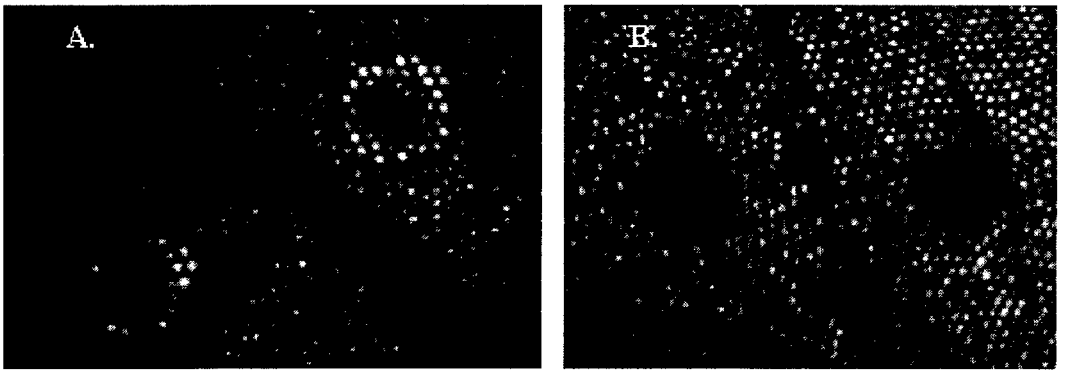


Figure 5
A. The photoreceptors of the acute snake eye lesion. A central core of destroyed cells, surrounded by a matrix of cells damaged and distinct, but still structurally intact. Those cells, initially destroyed, are unlikely to be salvageable by any possible treatment, but those in the matrix of injured cells may respond to treatment. **B.** The same eye, 3 weeks later. Many of the photoreceptors which were damaged are now gone. Saving some of these of photoreceptors is goal of the treatment strategy.

In an attempt to determine if oxidative stress (OS) was correlated to the ultimate death of these damaged photoreceptors, the dyes 2',7'-dichloro dihydrofluorescein diacetate (Molecular Probes D-399) and 5-(and-6)-carboxy-2',7'-dichloro dihydrofluorescein diacetate (C-400) were administered, by intra-cardiac injection, 15 minutes post laser exposure. Both of these dyes become fluorescent in the presence of reactive oxygen species, and have been used in *in vitro* experiments as biochemical markers for reactive oxygen species (ROS) and OS.[12,13] Imaging with the SLO's fluorescein filter, after laser exposure and systemic application of these indicators of ROS, we found areas of the lesion sites strongly marked by the dye's fluorescence, indicating the active presence of ROS and OS (Figure 6).

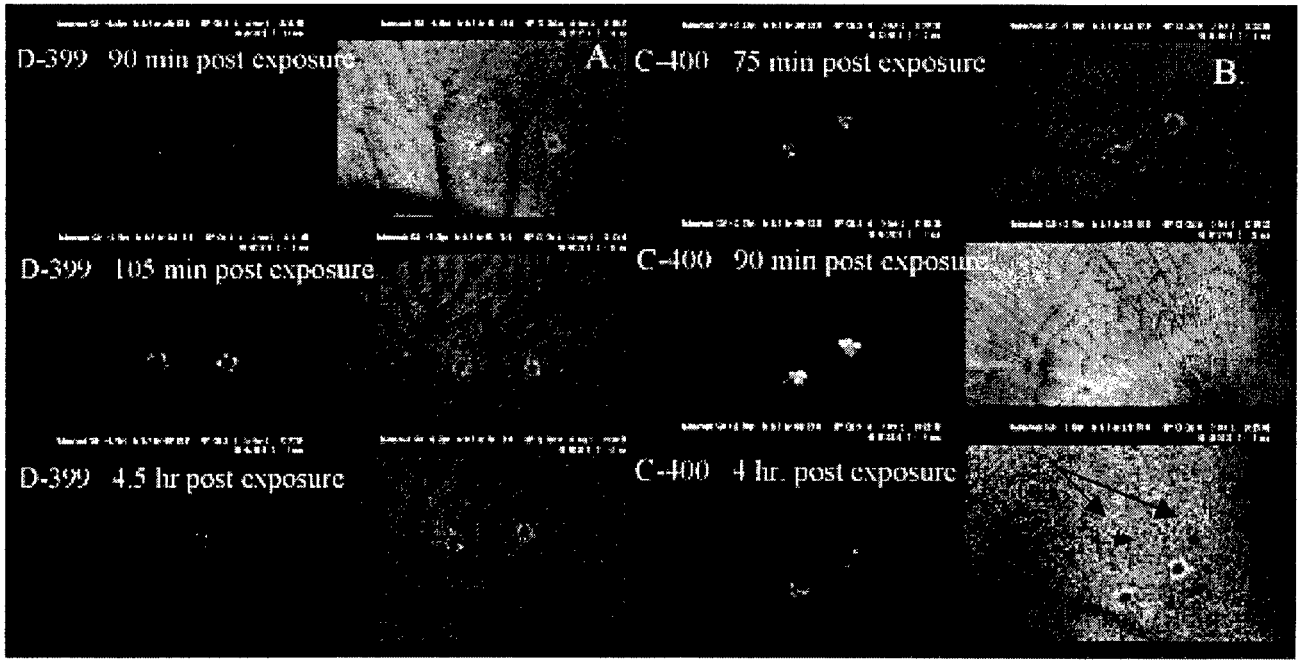


Figure 6
Fluorescent images on the left, same lesions imaged without fluorescein filter on the right. Note lack of staining at sites of old lesions (arrows) in B.

The dye 5-(and-6)-carboxy-2',7'-dichloro dihydrofluorescein diacetate (C-400), also illustrates the cellular immune response to laser injury in the retina (Figure 7) clearly staining white blood cells migrating to the injury site.

Figure 7
Oxidative stress and immune response to acute laser injury.

When we applied a thiol antioxidant, N-acetylcysteine (NAC) systemically, after the lesion but before the dyes, the fluorescence associated with the laser injury was reduced or absent (Figure 8). The treatment experiment that follows is an attempt to determine if the quenching effect we observed in the dye applications would translate to a sparing of the photoreceptors damaged but not initially destroyed by thermal laser lesions.

NAC is an antioxidant used as a standard in much of the OS research. It is also a therapeutic currently in use in clinical practice to treat a variety of diseases and injuries. In this pilot study, we attempted to determine whether the quenching effect on OS by the NAC administration, seen in the dye studies could be seen as a sparing of the cells injured but not immediately destroyed after laser injury. That is, the effects of a single dose treatment with the antioxidant, NAC, on temporal development of retinal laser injury.

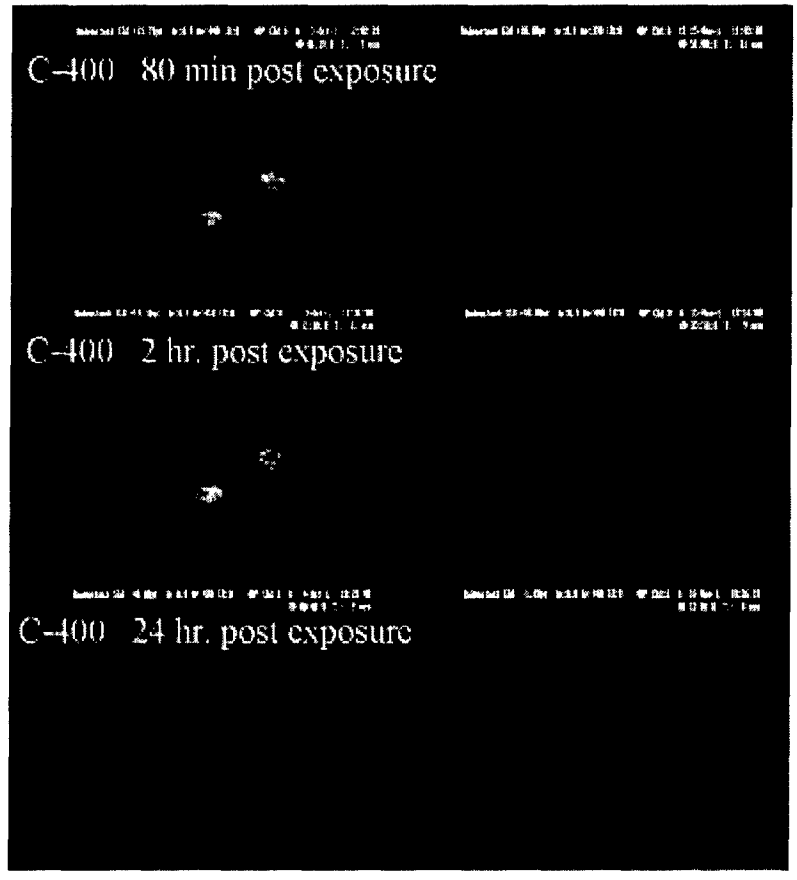


Figure 8
Effects of a single dose of N-acetylcysteine on fluorescence, induced by the dye C-400, in acute retinal laser injury. Dye alone on the left. Dye plus NAC on the right. In the first two time intervals, the NAC appears to quench the oxidative stress, but by 24 hours post, the lesion fluorescence in the two groups is similar.

Methods

All animals were treated in accordance with the Federal Animal Welfare Act and the Guidelines for the Care and Use of Laboratory Animals. The subjects, 12 Corn snakes (*E. g. guttata*, purchased from Glades Herps Inc.) approximately 3-4 feet in length, were kept in tagged, temperature controlled, escape proof, plastic cages lined with a disposable,

heavy-paper substrate. Water was available *ad lib,* in tip-resistant plastic cups, and each snake was fed one adult mouse per week. Before each exam session, subjects were anesthetized with a single intra-peritoneal (IP) dose of a ketamine / xylazine cocktail. The drugs were mixed in the ratio 3.5:1, ketamine: xylazine, and administered at 50-75 milligram (combined drugs) per kilogram of body weight.

In a placebo matched, crossover design, the subjects received 5 laser exposures per eye. Subjects randomly received either NAC, 150mg/kg, in sterile saline, or a matching amount of sterile saline, as a placebo, both injected IP. Half of the subjects received NAC or placebo 30 minutes post-exposure and half were treated 2-hours post-exposure. After a 3-week washout period, treatments (NAC vs. placebo) were reversed for each subject and the alternate eye received treatment. There were no unusual health effects noted during the study except that many of the snakes began their shedding cycles shortly after NAC treatment.

The laser exposures were made with a Nd:VO_4, DPSS, 532nm compact laser system (Intelite Inc., model GSF32-200p). The laser head and optics were mounted on and coaxially aligned with the SLO's imaging axis. Live SLO video images of the subject's retina were used to precisely place each exposure (Figure 9).

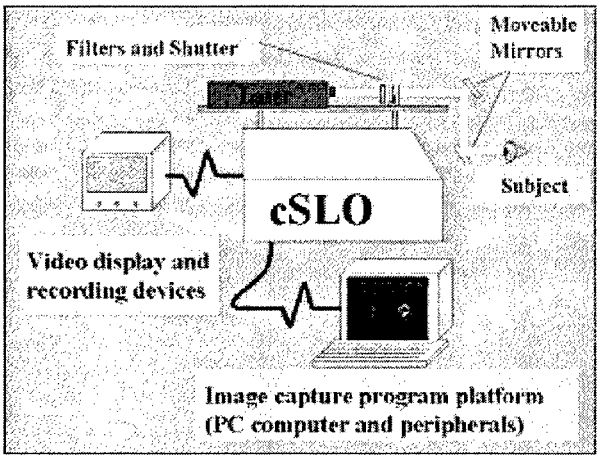


Figure 9.
Optical schematic of integrated examining instrument, image capture, and exposure system. CSLO; Rodenstock model 101, confocal scanning laser ophthalmoscope. Laser; Intelite Nd:VO_4 DPSS laser, 532nm, 200mW max power.

The shuttered continuous wave laser pulses were 20msec in duration, with the laser power regulated at 50-milliwatts, resulting in a 1-millijoule exposure. Laser power at the eye position was measured before and after each exposure session with a Newport laser power meter (model 1830C with a model 818 detector). Retinal spot size was approximately 50 μm. These laser exposures resulted in discrete, immediately visible lesions approximately 60-120 μm in diameter, in a linear array spaced approximately 300 μm apart in the superior temporal retina (Figure 10).

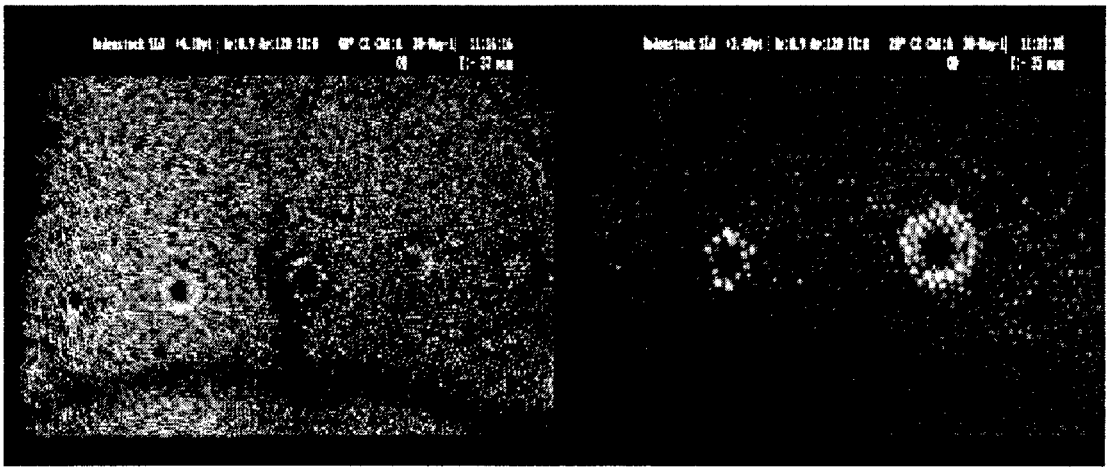


Figure 10
Thermal retinal lesion in the snake eye. Note the variable result of identical insult. Left image is a 40-degree field of view SLO image. All five lesions in the linear array are visible. Right, a 20 degree SLO image.

Data were collected from retinal images captured from SLO video. Retinal images were taken using a Rodenstock cSLO (model 101). Images were digital averages of 10-50 sequential frames (using the Image Record Module of the Rodenstock software; version 3.1), digitally recorded, and stored on the SLO control computer (PC). Recording times were: immediately pre-exposure, 1, 6, and 24 hrs post -exposure, and 3-weeks post-exposure. Each image stored contained two lesions, viewed at the high magnification setting of the SLO (20 degree field of view).

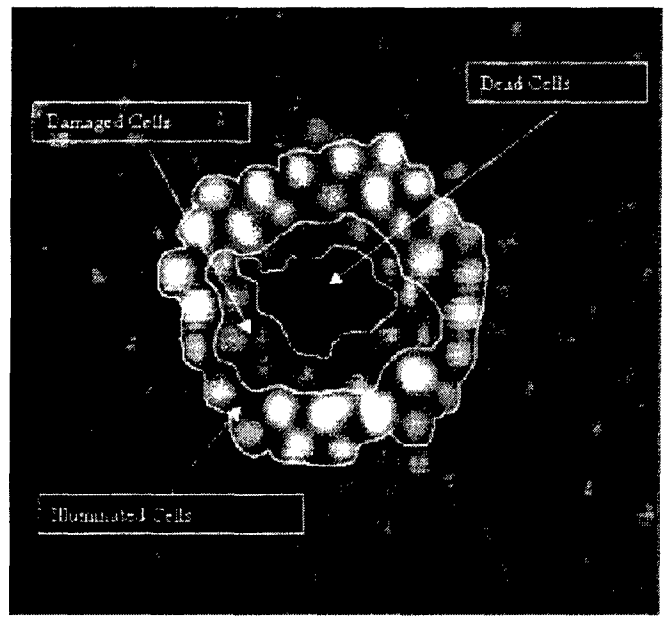
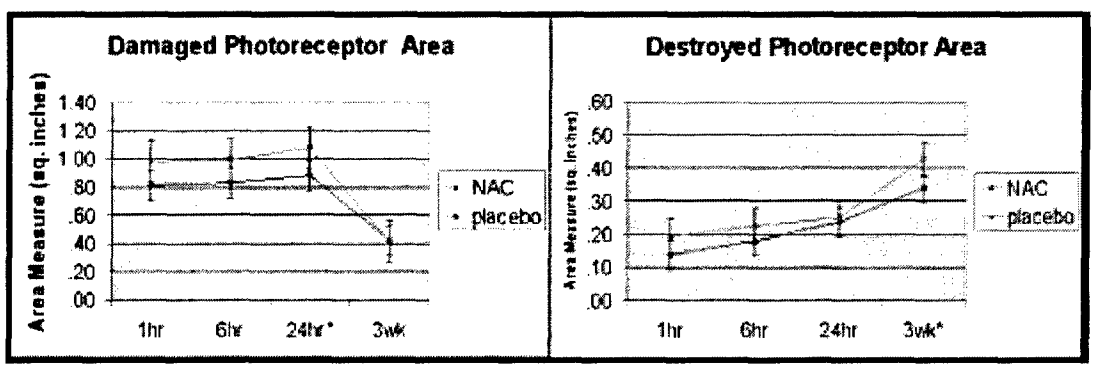


Figure 11
Examples of the areas raters were asked to measure. For analysis the areas labeled "Damaged" and "Illuminated" were combined as damaged area.

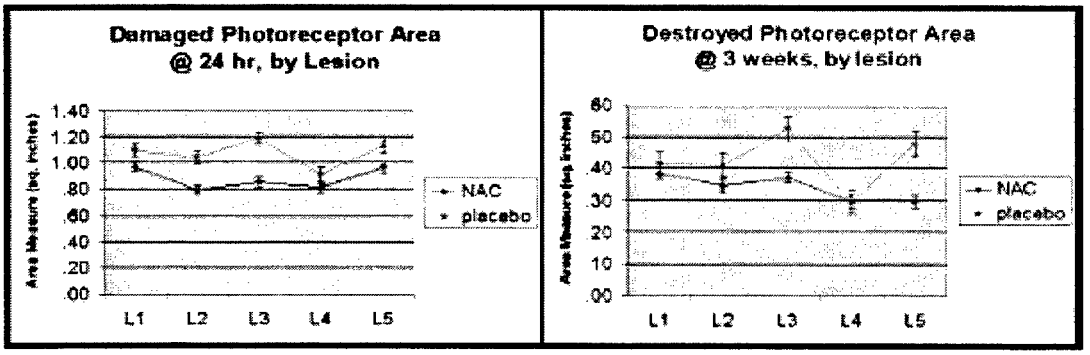
Images of each lesion from all four post exposure data collection times (1, 6, and 24 hours and 3 weeks) were measured by two raters, blind to the conditions of each image, using the public domain U.S. National Institutes of Health (NIH) Image program. Data collected consisted of measures of area and counts of missing and damaged photoreceptors (Figure 11).

Results

In the measurement of area affected, when all treated eyes were grouped together, a significant ($p \leq 0.01$) overall beneficial effect was present. In both damaged area (Graph 1) and counts of damaged cells at 24-hours (indicative of peak inflammatory response/OS), lesions in treated eyes were significantly smaller. The "destroyed" area (Graph 2) and cell numbers at 3-weeks (indicative of ultimate photoreceptor loss/survival), showed a similar sparing effect in the eyes of treated animals. The two different times of drug administration, 30 minutes post exposure and 2 hours post exposure, were not significantly different.



Graph 1 & 2
Plots of Damaged Area and Destroyed Area in treated and placebo eyes. An asterisk in the X-axis denotes significant difference between all treated eyes and all placebo eyes at the $p \leq 0.01$ level. Area is in square inches of screen space; 0.40 sq. inches is equivalent to 250 μm^2.



Graph 3 & 4

Plots of Damaged Area and Destroyed Area at the exam times found significantly different, plotted by lesion position. Note the general trend toward larger lesion size toward the center of the array (Lesion 3), perhaps a further demonstration of the known interaction on lesion development by adjacent lesions.

It is interesting to note the difference in lesion size by lesion position, and the effect of NAC treatment (Graph 3 & 4). L3 was the third lesion in a linear series of five, and thus the one most likely to show effects from adjacent lesions. It has been noted in this model and in other retinal laser injury studies that nearby lesions have a deleterious effect on one another. If the cause of this interaction is inflammatory immune response, and if antioxidants can diminish that inflammation, this may be of particular interest for follow-on research

Conclusions

This pilot experiment was an attempt to quantify data from the snake eye model and to investigate the role of oxidative stress in laser-induced retinal injury. The ability to observe cellular detail and quantify retinal damage with this model was confirmed. Methodological procedures for quantitative analysis of snake eye data are evolving, as is the use of molecular probes in the *in vivo* eye. The use of NAC, or similar antioxidants, shows promise as a potential therapeutic tool in this type of injury.

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